Protective Effect of *Phyllanthus fraternus* against Thioacetamide-Induced Mitochondrial Dysfunction

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**Summary** The effect of administration of thioacetamide on rat liver mitochondrial functions and the protective effect of an aqueous extract of *Phyllanthus fraternus* against thioacetamide-induced damage were studied. When rats were treated with thioacetamide, the rate of mitochondrial respiration was decreased significantly with both NAD+ linked and FAD linked substrates, and the respiratory control ratio, an index of membrane integrity and the P/O ratio, a measure of phosphorylation efficiency, decreased significantly. Also, there was a significant decrease in the activities of NADH dehydrogenase, succinate cytochrome *c* reductase, and cytochrome oxidase; whereas succinate dehydrogenase was not affected. A significant decrease was seen in membrane potential and in the level of mitochondrial ATP. There was a significant decrease in the levels of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin by thioacetamide treatment. The lipid peroxide level increased significantly in thioacetamide-treated rats. Administration of *P. fraternus* prior to thioacetamide treatment relieved the inhibition of all the parameters studied, and brought down the lipid peroxide levels significantly in liver homogenates and mitochondria. This study shows that *P. fraternus* protects against thioacetamide-induced toxicity by its ability to suppress the elevated lipid peroxide levels in the mitochondrial membrane.

**Key Words:** mitochondrial dysfunction, lipid peroxidation, *Phyllanthus fraternus*, oxidative phosphorylation, hepatotoxin

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Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; P/O, phosphate to oxygen ratio; RCR, respiratory control ratio; Tris, tris(hydroxymethyl) aminomethane; BSA, bovine serum albumin; SMP, submitochondrial particles; ANS, 1-anilino-8-naphthalene sulfonate.
Thioacetamide, a weak hepatocarcinogen, induces necrosis [1], cirrhosis, and tumors [2, 3] in the liver depending on the dosage and duration of treatment. Studies by Moller and Dargel [4] have shown that the structure and function of mitochondria are altered by chronic administration of thioacetamide to rats.

Mitochondrial function was shown to be disturbed in patients with cirrhosis of liver [5], and in rats treated with thioacetamide [4], CCl₄ [6], or ethanol [7]. Hepatotoxins such as CCl₄, thioacetamide, and ethanol are known to induce lipid peroxidation. Lipid peroxidation leads to membrane damage, which can be protected against by antioxidants present in the system. Under pathological conditions, however, these systems can be overwhelmed. Administration of nontoxic drugs would thus help to prevent deleterious effects of lipid peroxidation.

In the present study the acute effect of thioacetamide on liver mitochondrial function was investigated. Since there are no reports on prevention of the cytotoxicity induced by thioacetamide, an attempt was made to do so by using an aqueous extract of Phyllanthus fraternus, which is used in traditional medicine for the treatment of jaundice [8]. P. fraternus is a herb, up to 60 cm in height, and occurs as a winter weed throughout the hotter parts of India. The plant is bitter in taste and is used for gastric complaints and diabetes.

MATERIALS AND METHODS

Chemicals. ADP, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), cytochrome c, and 1,1,3,3-tetramethoxypropane were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from local firms.

Treatment of experimental animals. Male Wistar rats weighing 100±20 g were used for the present study, and were divided into four groups: 1) the control group, which received 0.2 ml of saline intraperitoneally; 2) the thioacetamide group, which received thioacetamide (25 mg in 0.2 ml saline/100 g BW), i.p., for four days, with sacrifice 24 h after the last dose; 3) the P. fraternus group, which received an aqueous extract equivalent to 100 mg dry powder of the plant in 0.5 ml of water/kg BW orally for five days; and 4) the P. fraternus + thioacetamide group, which received an aqueous extract of P. fraternus as described above and then thioacetamide (25 mg in 0.2 ml of saline) given for four days, with sacrifice 24 h after the last dose.

Isolation of mitochondria. Liver mitochondria were isolated by differential centrifugation according to the method of Lawrence and Davies [9]. The isolation medium consisted of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.2 mM EDTA, and bovine serum albumin (BSA) (36 mg/100 ml) adjusted to pH 7.4. The final pellet was suspended in 0.25 M sucrose to a protein concentration of 15-20 mg/ml. Protein was measured by the Biuret method [10], with BSA used as the standard. Solutions were prepared fresh daily in triple-distilled water. Submitochondrial particles (SMP) were prepared according to the method of Hackenbrock.

and Hammon [11]. The purity of the mitochondria was known from the activities of marker enzymes, and the maximum contamination by other subcellular fraction was up to 5%.

**Assay of oxidative phosphorylation.** Polarographic measurements of oxygen consumption were made with a Clark oxygen electrode in a Gilson 5/6 oxygraph. The reaction system (total volume, 1.7 ml) contained 50 mM Tris-Cl, 20 mM K<sub>2</sub>HPO<sub>4</sub>, 2 mM EDTA, 7 mM MgCl<sub>2</sub>, 50 mM sucrose, and 2.5–3 mg of freshly prepared mitochondria. After the addition of substrate (2 mM glutamate + 4 mM malate or 9 mM succinate) the state 3 respiration was initiated by the addition of 100–200 nmol of ADP. Rate of respiration, respiratory control ratio and phosphate to oxygen ratio (P/O) were calculated according to Estabrook [12]. For uncoupler-stimulated respiration, 0.1 μM CCCP was added.

**Enzyme assays.** Spectrophotometric assays of the respiratory enzymes were conducted with a Shimadzu-160A spectrophotometer.

NADH dehydrogenase activity was measured at 420 nm with potassium ferricyanide used as electron acceptor [13]. Activity of the enzyme was expressed as nmol of potassium ferricyanide reduced per min per mg protein.

Succinate dehydrogenase was assayed with phenazine methosulfate (PMS) and 2,6-dichloro phenolindophenol (DCPIP) used as electron acceptors. The reduction of DCPIP was followed at 600 nm [14]. The activity of the enzyme was expressed as nmol of DCPIP reduced per min per mg protein.

Cytochrome oxidase activity was measured by following the rate of oxidation of ferrocytochrome c at 550 nm [15]. The activity of the enzyme was expressed as nmol of cytochrome c oxidized per min per mg protein.

Succinate cytochrome c reductase activity was measured by following the reduction of ferricytochrome c at 550 nm [16]. Activity of the enzyme was expressed as nmol of cytochrome c reduced per min per mg protein.

Reverse electron transport in SMP was assayed as described earlier [17]. Activity of the enzyme was expressed as nmol of NAD reduced per min per mg protein.

Mitochondrial swelling was followed by change in absorbance at 520 nm [18]. Alanine amino transferase and aspartate aminotransferase were assayed in plasma and liver of control and experimental animals as described earlier [19].

Tissue content of adenine nucleotides was measured by standard enzymatic methods as described earlier [20]. Metabolite assays were coupled to an appropriate reaction (NAD(P)- or NAD(P)H-dependent dehydrogenases), and the change in absorbance was followed at 340 nm. Adenylate energy charge was calculated by the following equation, as proposed by Atkinson [21]: Energy charge = \((ATP + \frac{1}{2} ADP)/(ATP + ADP + AMP)\).

Membrane potential (Δψ) in SMP was measured by the distribution of the fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) across the submitochondrial membrane [22]. The difference in ANS fluorescence in submitochondrial particles between the energized and deenergized state was used for the
calculation of membrane potential. Membrane potential was expressed in millivolts.

Lipid peroxide level was determined in liver homogenates and mitochondria of control and treated animals according to the method of Ohkawa et al. [23]. Tetramethoxypropane was used as an external standard.

The concentrations of cytochromes aa₃, b, and c were determined from a difference spectrum (dithionite-reduced minus oxidized) recorded at room temperature with a Hitachi double beam spectrophotometer [24].

Mitochondrial lipids were separated by the procedure of Bligh and Dyer [25]. Phospholipids were separated by thin-layer chromatography using chloroform : methanol : water (65 : 25 : 4) as solvent, and the subclasses of phospholipids were detected by exposure to iodine vapor, followed by comparison with authentic standards. The individual phospholipids were digested with 60% perchloric acid, and the inorganic phosphate was estimated by the Fiske-Subbarow’s method [26].

Statistical analyses were conducted by Student’s t-test, and a p value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

The effect of thioacetamide administration on mitochondrial function and the protective effect of an aqueous extract of P. fraternus on thioacetamide-induced toxicity were studied.

The activities of transaminases (alanine and aspartate transaminases), which are used as marker enzymes for liver damage, were measured in the liver and plasma of control and thioacetamide-treated rats. In experimental rats alanine aminotransferase significantly decreased, by 56%, in the liver, whereas the activity in the plasma significantly increased by three fold (311%). Aspartate aminotransferase significantly decreased (by 63%) in the liver which was accompanied by a significant increase (575%) in the plasma (Table 1). It is clear from the results shown in Table 1 that the liver was damaged by administration of thioacetamide.

NADH oxidase, which gives information on the ability of transfer of electrons through all three sites of the electron transport chain, was measured with glutamate + malate as substrates. State 3 respiration decreased by 59%, while RCR

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver transaminases</th>
<th>Plasma transaminases</th>
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<tbody>
<tr>
<td></td>
<td>A1AT</td>
<td>AAT</td>
</tr>
<tr>
<td>Control</td>
<td>18 ± 2</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>8 ± 1*</td>
<td>31 ± 2**</td>
</tr>
</tbody>
</table>

Activities of transaminases are expressed as μmol of NADH oxidized per h per mg protein. A1AT, alanine amino transferase; AAT, aspartate amino transferase. Values are the mean ± SD for six animals. *p < 0.005; **p < 0.001 vs. corresponding control.

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and P/O were decreased by 80 and 99%, respectively, in thioacetamide-treated rats. Administration of an aqueous extract of P. fraternus prior to thioacetamide relieved the inhibition of state 3, respiratory control ratio (RCR), and P/O by 76, 59, and 86%, respectively (Table 2).

Succinate oxidase, which gives information on the rate of electron transfer through sites II and III, was decreased by 47% in thioacetamide-treated rats; whereas RCR and P/O were decreased by 73 and 49%, respectively. Administration of P. fraternus extract prior to thioacetamide relieved the inhibition on state 3, RCR, and P/O ratio were 80±6, 4.06±0.05, and 1.96±0.04, respectively, when succinate was used as substrate. Values are the mean ± SD for eight animals. *p < 0.005; **p < 0.001 vs. corresponding control.

<table>
<thead>
<tr>
<th>Group</th>
<th>State 3 respiration</th>
<th>RCR</th>
<th>P/O</th>
</tr>
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<tbody>
<tr>
<td>With glutamate + malate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±12</td>
<td>100±4.5</td>
<td>100±2.5</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>41±4*</td>
<td>20±1**</td>
<td>1±0.1**</td>
</tr>
<tr>
<td>P. fraternus</td>
<td>93±3</td>
<td>87±2</td>
<td>96±1</td>
</tr>
<tr>
<td>P. fraternus + thioacetamide</td>
<td>86±3</td>
<td>67±4</td>
<td>86±1</td>
</tr>
<tr>
<td>With succinate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±7</td>
<td>100±1</td>
<td>100±2</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>53±7*</td>
<td>27±1**</td>
<td>51±1**</td>
</tr>
<tr>
<td>P. fraternus</td>
<td>94±4</td>
<td>103±1</td>
<td>97±2</td>
</tr>
<tr>
<td>P. fraternus + thioacetamide</td>
<td>75±1</td>
<td>70±8</td>
<td>90±1</td>
</tr>
</tbody>
</table>

All the values are expressed relative to the control, which was taken as 100. The control value for state 3ADP respiration, RCR, and P/O was 73±9, 5.2±0.24, and 3.03±0.02, respectively, when glutamate+malate were used as substrates. The state 3ADP respiration, RCR, and P/O ratio were 80±6, 4.06±0.05, and 1.96±0.04, respectively, when succinate was used as substrate. Values are the mean ± SD for eight animals. *p < 0.005; **p < 0.001 vs. corresponding control.

In thioacetamide-treated rats, NADH dehydrogenase activity decreased by 61%, showing that the transfer of electrons from NADH to the prosthetic group of the flavoprotein [E-FMN to E-FMNH₂] was inhibited. Administration of an aqueous extract of P. fraternus prior to thioacetamide relieved the inhibition on NADH dehydrogenase by 79% (Table 3).

Succinate cytochrome c reductase, which gives information about site II of the electron transport chain, was decreased by 54% in thioacetamide-treated rats. Cytochrome oxidase, which is involved in the transfer of electrons from ferrocytochrome c to molecular oxygen, was decreased by 37% in the same rats. Pretreatment
with *P. fraternus* relieved the inhibition of succinate cytochrome c reductase and cytochrome oxidase by 44 and 70%, respectively. Succinate dehydrogenase did not show any change either by thioacetamide alone or thioacetamide along with *P. fraternus* extract (Table 3).

Reverse electron transport from succinate to NAD+, which gives information about site I of the electron transport chain, was significantly decreased, by 53% (Table 4).

Mitochondria from thioacetamide-treated rats showed a significant swelling compared with the control organelles (Fig. 1). Loss in the selective permeability control of mitochondria leads to swelling due to the excess transfer of solutes. The absorbance of mitochondrial suspension decreases as the mitochondrial volume increases. Soussi et al. [27] reported that increased permeability of the mitochondrial membrane leads to uncoupling of oxidative phosphorylation.

The lipid peroxide level, an index of membrane damage, increased by 62% in liver homogenates, and by 31% in mitochondria, of thioacetamide-treated rats. Administration of *P. fraternus* extract alone did not show any significant effect on the lipid peroxide level. Pretreatment with *P. fraternus* extract prior to thioacetamide lowered the stimulation in peroxide level from 62 to 27% and from 31 to 8% in homogenates and mitochondria, respectively (Table 5). All the complexes of mitochondrial electron transport chain have been shown to be vulnerable to damage by oxygen free radicals *in vitro* [28].

The concentrations of cytochrome *aa₃* and *b* decreased by 24 and 22%,
respectively, in thioacetamide-treated rats; whereas the decrease in the concentration of cytochrome c was insignificant, i.e., 12% (Table 6). The decrease in the level of cytochrome aa₃ and of b induced by thioacetamide was relieved by 67 and 32%, respectively, by pretreatment with *P. fraternus*. As cytochrome aa₃ is involved in the transfer of electrons from cytochrome c to molecular oxygen, a decrease in its concentration can inhibit the rate of electron transfer through this site.

Table 5. Effect of administration of thioacetamide with or without pretreatment with *P. fraternus* extract on lipid peroxide levels in liver homogenates and mitochondria.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lipid peroxide level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver homogenates</td>
</tr>
<tr>
<td>Control</td>
<td>130±10</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>210±22**</td>
</tr>
<tr>
<td><em>P. fraternus</em></td>
<td>145±9</td>
</tr>
<tr>
<td><em>P. fraternus</em> + thioacetamide</td>
<td>165±21</td>
</tr>
</tbody>
</table>

Level of lipid peroxide is expressed as nmol malondialdehyde formed per 100 mg protein. Values are the mean ± SD of eight animals. *p <0.05; **p <0.005 vs. corresponding control.
of phosphatidylcholine, phosphatidylethanolamine, or cardiolipin. In *P. fraternus*-pretreated rats, the drop in phosphatidylcholine, phosphatidylethanolamine, and cardiolipin was reversed by 74, 68, and 82%, respectively (Table 7). Phosphatidylcholine and phosphatidylethanolamine have been shown to be essential for the proper functioning of cytochrome oxidase in ethanol-fed baboons [29]. Cardiolipin, due to its high degree of unsaturation, is prone to lipid peroxidation under oxidative stress. Cytochrome oxidase activity is dependent on cardiolipin for maximum activity [27]. In the present study the decrease in cytochrome oxidase activity would be due to decreases in cardiolipin and cytochrome aa₃ contents.

Table 8 shows the effect of thioacetamide on the energy charge of the cell; i.e., there was a significant decrease in the level of ATP (57%), and a significant increase in the level of AMP (204%) in the thioacetamide-treated rats compared with the control levels. No change was observed in the ADP level. The energy charge of the cell was decreased significantly (24%) in thioacetamide-treated rats. Membrane potential ($\Delta \psi$), the main component of proton motive force [30], decreased significantly, by 24%, in the treated rats (Table 3). It was reported earlier that membrane potential is decreased by changes in mitochondrial lipid and protein composition [31–33]. Any decrease in the membrane potential can affect ATP synthesis, since this is the major driving force for the formation of ATP from ADP.

Our studies show that acute administration of thioacetamide to rats leads to mitochondrial dysfunction. Increased lipid peroxide level in thioacetamide-treated rats caused a loss of membrane integrity of mitochondria, decreasing the rate of electron transfer through the respiratory chain. Castilho et al. [34] showed that in vitro lipid peroxidation of mitochondria causes a decrease in membrane potential and alterations in membrane fluidity. Administration of an aqueous extract of *P. fraternus* prior to thioacetamide relieved the inhibition on all the mitochondrial enzymes studied. An aqueous extract of the leaves of *P. fraternus* has been shown to be effective in the treatment of jaundice [35] and diabetes [36].

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REFERENCES


